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Towards Developing HIV-2 Lentivirus-Based Retroviral Vectors for Gene Therapy: Dual Gene Expression in the Context of HIV-2 LTR and Tat

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Because of the distinct ability of retroviruses to integrate into the target cell genome and thus achieve long-term expression, retrovirus vectors hold great promise for stable gene transfer. Such vectors derived from human immunodeficiency retroviruses (HIVs) and other lentiviruses are envisioned to possess several advantages, especially for in vivo gene therapy of HIV infection and acquired immunodeficiency syndrome (AIDS) where targeting CD4+ T cells/macrophages and pluripotent non-dividing stem cells would be required. Among these is the ability of HIVs to transduce nondividing cells in contrast to the murine retroviruses which require target cell mitosis. The advantages of the lentivirus vectors will be further enhanced by the development of multigenic vectors carrying more than one gene in a dependent or independent transcriptional unit. Separate from the issue of transduction efficiency, information is needed about the impact of the configuration of the genes in a multigenic vector on their expression. Towards this end, we investigated the expression of genes specifically directed by the HIV-2 LTR and Tat in a prototypic minimal transfer vector. We found that the expression of a gene in a dual gene configuration depended upon its position in the transcriptional unit and that the insertion of an internal translational initiation mechanism improved the expression of the downstream gene. Apparently not sufficiently appreciated previously, these effects were promoter and cell-type dependent. Our data also suggest that the commonly used cellular or viral promoters may be orders of magnitude less effective than HIV-2 LTR in the presence of Tat, and thus may not be useful as internal promoters in the context of the HIV-2 LTR:Tat regulatory loop. ***J. Med. Virol. 54: 118–128, 1998.*** © 1998 Wiley-Liss, Inc.†

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INTRODUCTION

Paucity of available vectors for gene therapy mandates continuing developmental effort. While transduction efficiency of a vector is an important issue, equally important is the knowledge of factors governing expression of the gene(s) carried by the vector [Anderson, 1992; Mulligan, 1993; Crystal, 1995; Arya and Gallo, 1996a]. Retroviral vectors for gene transfer have been under development for several years [Gilboa et al., 1986; Miller and Rosman, 1989]. In order to aim at multiple targets or to achieve multiple purposes, it is desirable to design retroviral vectors carrying several genes. The design of such multigenic vectors requires information about such issues as the effect on expression by the (i) order of the placement of two or more genes in a transcriptional unit (positional effects), (ii) choice of the promoter, primary or internal (promoter effects), (iii) transcription of one or more genes as part of the same or different transcriptional unit (unit effects), (iv) transcription from the same or opposite DNA strand (polarity effects), and (v) independent internal initiation of translation in a polycistronic message (cistron effects).

Some studies addressing these issues individually or in a limited combination with simple murine retroviral vectors have been reported in recent years, but these vectors have not lived up to their promise. Nonetheless, these studies provide guidelines for future investigations. For example, the presence of a gene upstream of a second gene in a murine retroviral transcriptional

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unit was reportedly detrimental to the expression of the downstream gene in transduced murine hematopoietic cells and that the inclusion of an internal promoter only modestly improved the expression of the downstream gene in hematopoietic cells [Bowtell et al., 1988]. Comparison of internal promoters revealed that the strength of the promoter depended not only on its structure but also on its target cell in an unpredictable manner [Li et al., 1992; Correll et al., 1994]. The placement of a picornaviral sequence called internal ribosomal entry site (IRES) between two genes within a murine retroviral transcriptional unit may enhance the translation of the downstream gene [Ghattas et al., 1991; Morgan et al., 1992; Chen et al., 1993].

Our effort is directed towards developing HIV-2-derived retroviral vectors [Arya et al., 1994; Garzino-Demo et al., 1995; Arya and Gallo, 1996b]. The present study focuses on the design of HIV-2 long terminal repeat (LTR)-based vectors. These vectors are likely to possess a number of advantages, particularly for gene therapy of HIV infection and acquired immunodeficiency syndrome (AIDS). These include tropism for human CD4+ cells, targets of HIV infection in vivo, regulated expression modulated by the regulatory gene-response element loops, and vector DNA integration for likely persistent expression. An important advantage of these vectors is their ability to affect expression in nondividing cells including stem cells [Akkina et al., 1996; Naldini et al., 1996]. Such vectors combined with packaging systems could be used for ex vivo gene delivery and the vectors themselves for DNA vaccination by nonviral procedures. It should be recognized that because of the limited basal promoter activity of the LTR, HIV-based transfer vectors will need to be provided with Tat in *cis* or in *trans*. This could be achieved by either carrying the *tat* gene on a separate vector or making it a part of the vector itself. For the use of HIV-2 vectors in infected individuals in AIDS, Tat will be available in the target cell as part of the gene delivered by the infecting virus, thus making the vector a part of the autoregulatory loop. The expression of the transferred gene will be high only when Tat is available. This will minimize any toxic effects of the transferred gene whether transduced into target or nontarget cells. In the present study, we have utilized HIV-2 *tat* gene both as a transactivator as well as a model gene along with the bacterial *neo*-resistance gene as the second model gene. Direct DNA-mediated transfection was employed to circumvent the complications arising from the effect of vector modification on transduction efficiency. We present here our results on the effect on the expression of the relative positioning of two genes in HIV-2-derived expression clones with or without the provision for independent translational initiation of the bicistronic message and compare Tat-transactivated LTR-directed expression with that directed by a different viral (CMV) and cellular (β-actin) promoter.

MATERIALS AND METHODS

Molecular Cloning

The basic HIV-2 LTR clone, termed pSGT-3, was derived from a biologically active and noncytopathic molecular clone of HIV-2 (ST isolate) [Kumar et al., 1990; Arya and Sadaie, 1993]. It contained both the 5′-LTR and the 3′-LTR linked together with a short intervening sequence consisting of a part of the 5′-leader sequence with the major splice donor site and a sequence upstream of the 3′-LTR but lacking the *nef* gene initiator codon. The clone pSGT-3 (SL) contained a synthetic linker consisting of multiple cloning sites at the junction of 5′ and 3′ LTR. To obtain desired molecular clones, genes or regulatory elements were inserted at this multiple cloning site. HIV-2 *tat* gene was derived from a cDNA clone of HIV-2(ST) [Arya, 1993; Arya and Mohr, 1994] and was modified to contain only the *tat* open reading frame with the deletion of the *rev* open reading frame. It also contained 5′-untranslated leader sequence of the cDNA clone. Bacterial *neo*-resistance gene (*neo*) for the LTR-based *neo* clones was derived from the molecular clone (pLZIN) kindly provided by I. R. Ghattas [Ghattas et al., 1991]. The human cytomegalovirus (CMV) promoter based clones were derived from the pCMV-*tat* clone previously described [Arya, 1993; Arya and Mohr, 1994]. The gene or the cassette was inserted into the basic pSGT-3(SL) LTR clone by using synthetic linkers corresponding to the restriction site of the multiple cloning site. The resulting clones were characterized by detailed restriction mapping and checked by partial DNA sequencing. The genetic manipulations were carried out by the standard procedures of molecular cloning.

DNA-Mediated Transfection

For transient expression, human lymphatic CEM cells were transfected by the DEAE-dextran protocol as described [Arya and Gallo, 1988; Garzino-Demo et al., 1995]. About 8×10^6 cells were cotransfected with the 4 μg of indicator HIV-2 LTR-CAT (chloramphenicol acetyltransferase gene) DNA (clone pSLTR-CAT) [Arya, 1990; Arya and Mohr, 1994] and 1 μg of the test DNA containing the *tat* and/or *neo* gene. Cells were harvested 40–48 hr after transfection and processed to obtain 200 μl of the cytoplasmic extract. For obtaining long-term transfected CEM cells, about 6×10^6 cells were suspended in 0.25 ml of the serum-free culture medium containing 6 μg plasmid DNA and pulsed for 50 msec at 230 V and 80 μF with a Gibco/BRL Cell Porator system (Life Technologies, Gaithersburg, MD). After culturing the transfected cells in the complete medium for 48 hr, drug G418 (Geneticin, Gibco/BRL) was added to a concentration of 400 μg/ml. The cells were subsequently cultured and subcultured in the presence of G418.

Simian epithelioid COS cells were transfected by the calcium phosphate protocol [Arya, 1993]. About 1×10^6 early passage COS cells were transfected with 10 μg of the test DNA containing *tat* and/or *neo* gene and with

or without 10 μg of the indicator pSLTR-CAT DNA. Cells were processed to obtain 100 μl of the cytoplasmic extract.

Measurement of Tat and Neo Activity

Tat activity was evaluated by determining the ability of the *tat* gene to transactivate HIV-2 LTR-directed CAT gene expression in transiently transfected human lymphocyte CEM and simian epithelioid COS cells. Aliquots of the cytoplasmic extract (10–100 μl) of the cotransfected cultures were incubated for various times with [^{14}C]chloramphenicol and acetyl-CoA to measure the induction of the CAT gene expression by quantitating the conversion of chloramphenicol to its acetylated forms separated by thin layer chromatography and scintillation counting or by Phosphor-Imager analysis. Typically, for CEM cells transfected with clones lacking *tat* gene, 100 μl of cell extract (equivalent to 4×10^6 cells) was incubated for 4 hr, while for cells transfected with the clones containing *tat* gene, it was generally sufficient to incubate 10 μl of cell extract (equivalent to 0.4×10^6 cells) for 10 min to obtain measurable substrate conversion. Often two or three different assay conditions were used to ensure measurements in the linear range. As COS cells grew in monolayer, protein concentration of the cytoplasmic extracts was first determined, and equivalent amounts of extracts were used for CAT assays. Typically, the assays used 1–10 μl of extract (equivalent of $1–10 \times 10^5$ cells) incubated for 30 min.

Neo gene expression was analyzed by the enzyme-linked immunosorbent assay (ELISA) with reagents obtained from 5 Prime–3 Prime, Inc. (Boulder, CO). Briefly, microtiter plates were coated with neomycin phosphotransferase II (NPTII) antibody followed by incubation with a solution of bovine serum albumin. Serial dilutions of the cytoplasmic extract of transfected cultures or NPTII enzyme standard (range: 30 to 1,000 pg/ml) were applied to the microtiter plate wells. The biotinylated antibody to NPTII was then added, followed by the addition of avidin-horseradish peroxidase. The bound horseradish peroxidase was allowed to react with its substrate (TMB-H_2O_2) and reaction products quantitated calorimetrically. Whenever applicable, cytoplasmic extracts were normalized by measuring their protein content by the calorimetric protein assay (Bio Rad, Richmond, CA). Luciferase gene expression was determined by using the assay kit supplied by the Promega Corporation (Madison, WI) utilizing a luminometer to measure the intensity of the generated light.

The data in this report summarize the results of two or more separate experiments. Replicate experiments were done with cells at different passages and represent independent transfections.

In Situ Hybridization

Cells were processed for in situ hybridization with antisense *tat* and *neo* gene probes by the standard procedure [Mitchell et al., 1992; Kashanchi et al., 1997]. Briefly, cells were air-dried on Teflon-coated glass slides, fixed with 40% formaldehyde, and hybridized with specific probes. When necessary, slides were stored at −20°C under 70% ethanol. Digoxigenin-labeled antisense *tat* and *neo* probes were obtained by in vitro transcription of riboprobe vectors containing *tat* and *neo* gene translation open reading frames in the reverse orientation to the T3 RNA polymerase promoter. The transcripts were reduced in size to about 300 nucleotides by controlled alkaline hydrolysis and purified by chromatography on Sephadex G50 followed by ethanol precipitation. The slides were assayed in duplicate receiving amounts of probe predetermined to be optimal for hybridization with minimal background noise.

RESULTS

To determine the effect of the position of a gene on its expression in a dual gene configuration, a set of molecular clones were initially constructed containing the indicator CAT and *neo* genes in reciprocal upstream and downstream positions. The expression of these clones in human T-lymphocytic CEM cells was evaluated by analyzing CAT gene expression as previously described [Arya, 1993; Arya and Gallo, 1988; Arya and Mohr, 1994; Arya and Sadaie, 1993]. While the basal level expression of the CAT gene in the absence of Tat was low, its expression in the presence of Tat could be meaningfully compared (Fig. 1). Apparently, the CAT gene, when placed in the upstream position (clone LTR-CAT-*neo*), was expressed more favorably than when placed in the downstream position (clone LTR-*neo*-CAT). Because of the regulatory role of the *tat* gene in HIV infection and the possible desirability of including it in a general gene therapy vector and to obviate the necessity of providing it through a separate vector, we next constructed dual gene clones with *tat* and *neo* genes as indicator genes. We reasoned that this would allow an analysis of the autoregulatory aspects of the LTR: Tat interaction. We thus analyzed the expression of the *tat* gene in these clones by measuring its ability to transactivate HIV-2 LTR-linked CAT gene expression in trans (Fig. 2). The expression of the *tat* gene in the upstream position (clone LTR-*tat*-*neo*) was about 300-fold higher compared to its expression in the downstream position (clone LTR-*neo*-*tat*) in these experiments. The importance of the position of the gene on its expression was further confirmed by studying the dose-responses of the two representative clones, one with *tat* gene in the upstream position (clone LTR-*tat*-*neo*) and the other with *tat* gene in the downstream position (clone LTR-*neo*-*tat*) (Fig. 3). Clearly, the response was dose-dependent and differential transactivation was maintained over the range of transactivator DNAs used.

The differential expression of the genes was not due to any variation in the transfection efficiencies of the individual clones. They were similar in structure, and experimentally, cotransfection of these clones with a different indicator clone, that is, luciferase gene linked

Vector			CAT gene expression	
			% AcCm	Relative
LTR-CAT-*neo*	LTR–CAT–NEO–LTR	+ None	0.3 ± 0.1	1.0
LTR-CAT-*neo*	LTR–CAT–NEO–LTR	+ pCM-*tat*	94 ± 6	495 ± 250
LTR-*neo*-CAT	LTR–NEO–CAT–LTR	+ pCM-*tat*	0.6 ± 0.1	1.4 ± 0.3

Fig. 1. Expression of CAT gene in HIV-2 LTR-based dual gene retroviral vectors in human lymphocytic CEM cells in the presence or absence of HIV-2 Tat. About 8×10^6 cells were cotransfected with 4 μg of vector and 1 μg of Tat DNA by the DEAE-dextran protocol. About 40 to 48 hr posttransfection, the cells were harvested to prepare 200 μl of the cytoplasmic extract, and aliquots (10 to 100 μl) were incubated for variable lengths of time with [^{14}C]chloramphenicol (Cm) and acetyl CoA, and CAT enzyme mediated acetylation of chloramphenicol (AcCm) was quantitated by thin layer chromatography and scintillation counting. The results represent two or more independent transfections and are presented both as the percent of substrate (Cm) converted into the product (AcCm) and as relative expression. Expression of the HIV-2 Tat was directed by the CMV promoter in these experiments.

to the SV40 promoter, gave the same level of luciferase expression (not shown).

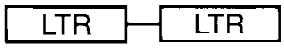
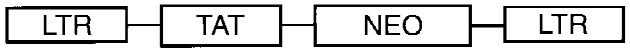
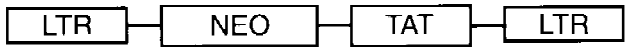
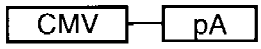
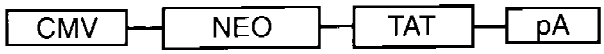
To determine if the positional effects were restricted to the HIV-2 LTR or were more universal, the effect of the position of the gene on its expression was studied in the context of a heterologous CMV promoter in place of the HIV-2 LTR promoter (Fig. 2). While the *tat* gene induced high levels of transactivation of the LTR-CAT gene expression when it was upstream of the *neo* gene (clone CM-*tat*-*neo*), this transactivation was much more modest when the *tat* gene was downstream (clone CM-*neo*-*tat*).

The expression of the *tat* gene in single- and dual-gene clones in epithelioid COS cells was also investigated (Fig. 4). Compared with the CEM cells, the *tat* gene encoded by the clone containing this gene alone (LTR-*tat*) appeared to be less effective in transactivating HIV-2 LTR-CAT gene expression in COS cells. In these cells, there was only a moderate effect on the position of the gene on its expression. Comparison of the clone LTR-*neo*-*tat* with clone LTR-*tat*-*neo* showed only two- to three-fold reduction in *tat* expression.

To simulate conditions relevant to ex vivo gene therapy approaches requiring cell selection, CEM cultures transfected with dual gene clones containing *neo* gene were subjected to G418 drug selection and examined for *neo* gene expression biochemically and by in situ hybridization. Analyses showed that the *neo* expression was higher when it was placed upstream than downstream of the *tat* gene (Fig. 5). Consistent with the biochemical data was the observation of the relatively more intense staining of cells in cultures transfected with vector with neo gene in the upstream than in the downstream position (Fig. 6). Clearly the positional effects were independent of the nature of the gene, whether *tat* or *neo*. Interestingly, there were higher numbers of *neo* positive cells that were less intensely stained in the LTR-*tat*-*neo* than in the LTR-*neo*-*tat* transfected cultures. This suggests that while positioning of the *tat* gene upstream favors its transcription, it hampers the effective translation of the downstream gene.

To improve the expression of the downstream gene in a multigenic vector the following two approaches can be envisioned: (i) provide a mechanism to initiate independent translation of the downstream open reading frame or (ii) include an internal promoter to affect independent transcription of the downstream gene. To test the first approach, we inserted the picornavirus IRES sequence between the *tat* and *neo* gene in the clone with the *neo* gene in the downstream position (clone LTR-*tat*-IRES-*neo*). Comparison of this clone with the corresponding clone lacking IRES element showed that the insertion of this element resulted in the improvement of the *neo* gene expression. Thus, independent initiation of translation of the downstream gene was beneficial. The improvement was, however, variable and this variability in part may be related to the state of the integration of the vector DNAs in different cells in the population.

To test the feasibility and validity of the use of an internal promoter to initiate independent transcription of the downstream gene within the context of HIV-2 LTR as a primary promoter in the presence of *tat*, the LTR activity was compared with that of a different viral (CMV) and a cellular (β-actin) promoter. Simulat-

Vector		CAT gene transactivation	
		% AcCm	Relative
LTR		0.7 ± 0.4*	1.0
LTR-*tat*		53.5 ± 28.3#	18,000 ± 5,800
LTR-*neo*		1.7 ± 1.9*	2.0 ± 2.7
LTR-*tat-neo*		37.3 ± 17.2#	14,700 ± 7,700
LTR-*neo-tat*		28.4 ± 14.5*	50 ± 26
CMV-O		1.1 ± 0.2*	1.0
CMV-*tat*		14.5 ± 5.3#	3,200 ± 1,400
CMV-*neo*		1.0 ± 0.2*	1.0 ± 0.2
CMV-*tat-neo*		20.1 ± 9.4#	4,100 ± 1,300
CMV-*neo-tat*		50.7 ± 11.2*	48 ± 12

* Assay parameters – 100 μl extract, 4 hr incubation
# Assay parameters – 10 μl extract, 10 min incubation

Fig. 2. Expression of *tat* gene in HIV-2 LTR and CMV promoter-based dual gene vectors in human lymphocytic CEM cells. Tat gene expression was quantitated by its ability to transactivate HIV-2 LTR linked to the indicator CAT gene (LTR-CAT) provided through a separate vector by cotransfection. About 8×10^6 CEM cells were transfected with 1 μg of vector and 4 μg of indicator DNA by the DEAE-dextran protocol. Cells were processed to obtain 200 μl of the cytoplasmic extract, and aliquots (10–100 μl) of extracts were incubated for various lengths of time with [^{14}C] chloramphenicol and acetyl CoA. Typically for cultures transfected with clones containing the *tat* gene, 10 μl of the cytoplasmic extract was incubated for 10 min and, for cultures lacking the *tat* gene, 100 μl of the cytoplasmic extract was incubated for 4 hr. The conversion of chloramphenicol (Cm) to acetylated chloramphenicol (AcCm) was quantitated by thin layer chromatography and scintillation counting. The results are presented both as a percent of substrate (Cm) converted into the product (AcCm) as well as relative expression. The results represent two or more independent transfections.

ing the conditions of HIV infection, HIV-2 provirus was used as a source of Tat in these experiments. Although the CMV promoter and, to a lesser extent, β-actin promoter gave higher basal level expression, the expression of the HIV-2 LTR in the presence of Tat was an order of magnitude higher than that of the CMV or β-actin promoter (Table I). There was some suppression of the CMV and β-actin promoter activity by the HIV-2 provirus.

DISCUSSION

A target-specific therapeutic retroviral vector capable of delivering a desired gene 'safely and effectively' remains unavailable. The preclinical studies presented here contribute towards our knowledge of the parameters influencing the expression of a transgene, specifically in human cells. Human and primate lenti-retroviruses, though complex in nature, provide a window of opportunity to develop well-targeted and regulated gene transfer vectors. They provide for receptor-directed target cell tropism and regulatory gene network-dependent ability for regulated and long-term expression. Uniquely, the lentiviruses such as HIV-1 and HIV-2 can transduce nondividing or postmitotic cells made possible by their possession of the R and X genes [Akkina et al., 1996; Corbeau et al., 1996; Naldini et al., 1996]. This contrasts to murine retroviruses that require target cell mitosis for transduction.

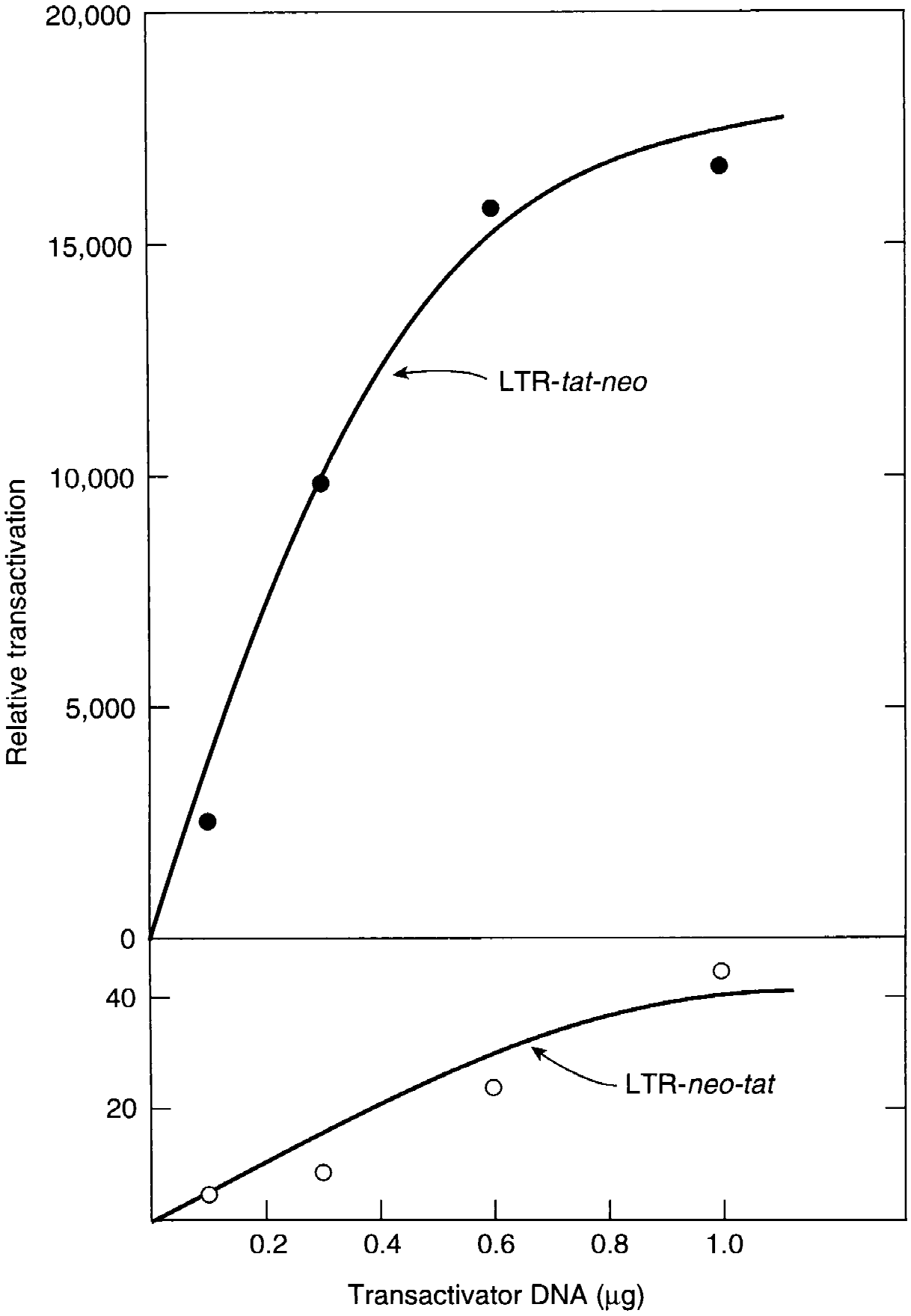


Fig. 3. Dose-response for *tat* gene expression by the HIV-2 LTR-based dual gene vectors in human lymphocytic CEM cells. *Tat* gene expression was assessed by measuring its ability to transactivate HIV-2 LTR-direct CAT gene expression in co-transfected CEM cells as described in the legend to Figure 2.

Vector		CAT gene transactivation	
		% AcCm	Relative
LTR	LTR — LTR	0.7 ± 0.4	1.0
LTR-*tat*	LTR — TAT — LTR	57 ± 23	98 ± 69
LTR-*neo*	LTR — NEO — LTR	0.3 ± 0.1	0.4 ± 0.2
LTR-*tat-neo*	LTR — TAT — NEO — LTR	52 ± 14	97 ± 62
LTR-*neo-tat*	LTR — NEO — TAT — LTR	21 ± 7	40 ± 30

Fig. 4. Expression of *tat* gene in HIV-2 LTR-based dual gene vectors in simian epithelioid COS cells. About 1 to 2 × 10^6 cells were transfected with 10 μg of vector and 10 μg of indicator DNA by the calcium phosphate protocol. Cytoplasmic extracts (100 μl) were prepared and their protein concentration was determined. Extract containing equivalent amounts of protein were incubated for 30 min to measure CAT gene expression as described in the legend to Figure 2.

Notably, the desirable nuclear targeting function and the undesirable cell cycle arrest function are segregated in HIV-2, being assigned to genes X and R, respectively [Fletcher et al., 1996]. In HIV-1 these two functions are encoded by the same gene (R), which makes it difficult to take advantage of one function without being encumbered by the other. Furthermore, HIV-2 vectors would be safer as they are not likely to recombine with the resident HIV-1 genetic information because of limited sequence homology.

Encouraged by the body of knowledge we and others have created for the Tat-mediated regulation of HIV-2 LTR (Arya and Gallo, 1988; Arya, 1990; Markovitz et al., 1990), we designed dual-gene vectors, some of which contained *tat* as both the transactivator and the indicator gene, while for others, it was provided in trans. Whether *tat* gene was in *cis* or in *trans*, the expression of the gene in a dual gene vector depended on its position, consistent with the scanning model of the expression of nonoverlapping open reading frames contained in a single transcriptional unit [Kozak, 1991]. Accordingly, the ribosomal initiation complex assembles at the cap site and transverses the length of the message initiating translation when it encounters an initiator codon and terminating it at the translational termination codon. In a polycistronic message, the translation occasionally can begin at the second initiator codon but the downstream open reading frame is translated much less efficiently. This would in part account for the reduced expression of *tat* gene when it was present downstream than upstream of the *neo* gene. Further, the translation efficiency is influenced by the context of the initiator codon, the optimal being (−3) purine, (−2) purine/pyrimidine, (−1) purine/pyrimidine-*ATG*-(+4) purine [Kozak, 1991]. However, this was not a factor here because both the genes had the optimal context; for Tat, GAA-*ATG*-G and for Neo, ACC-*ATG*-G [Arya, 1987].

The expression of the *neo* gene in long-term transfected culture was also differentially affected. In these cultures, the expression of the gene will not only be positionally dependent but also will be conditioned by the state of DNA integration. Because of the relative positioning of the *neo* and *tat* gene and Tat-mediated autostimulation, the expression of *tat* and *neo* genes will be affected differentially at the transcriptional and translational levels. The expression of the *tat* gene will be favored translationally and transcriptionally when present in the upstream position, but not favored by either mechanism in the downstream position. In contrast, the *neo* gene will be favored either translationally or transcriptionally depending on its position but not by both mechanisms.

Comparison of the CMV and LTR promoter revealed that transactivation levels and positional effects depended on the promoter, it being less marked for the CMV than for the LTR promoter. Apart from the inherent differences in the promoter activities, the Tat produced by LTR-*tat* vector will stimulate its own expression by feedback autostimulation. The positional effects will also be differentially affected by this autostimulation loop and thus will be promoter dependent. The expression of the *tat* gene will be affected by its position at the translational level and amplified by autocrine stimulation at the transcriptional level. The CMV promoter will be refractory to this feedback autostimulation at the transcriptional level. An additional possibility is the existence of cellular factors which preferentially enhance HIV-2 LTR but not CMV promoter.

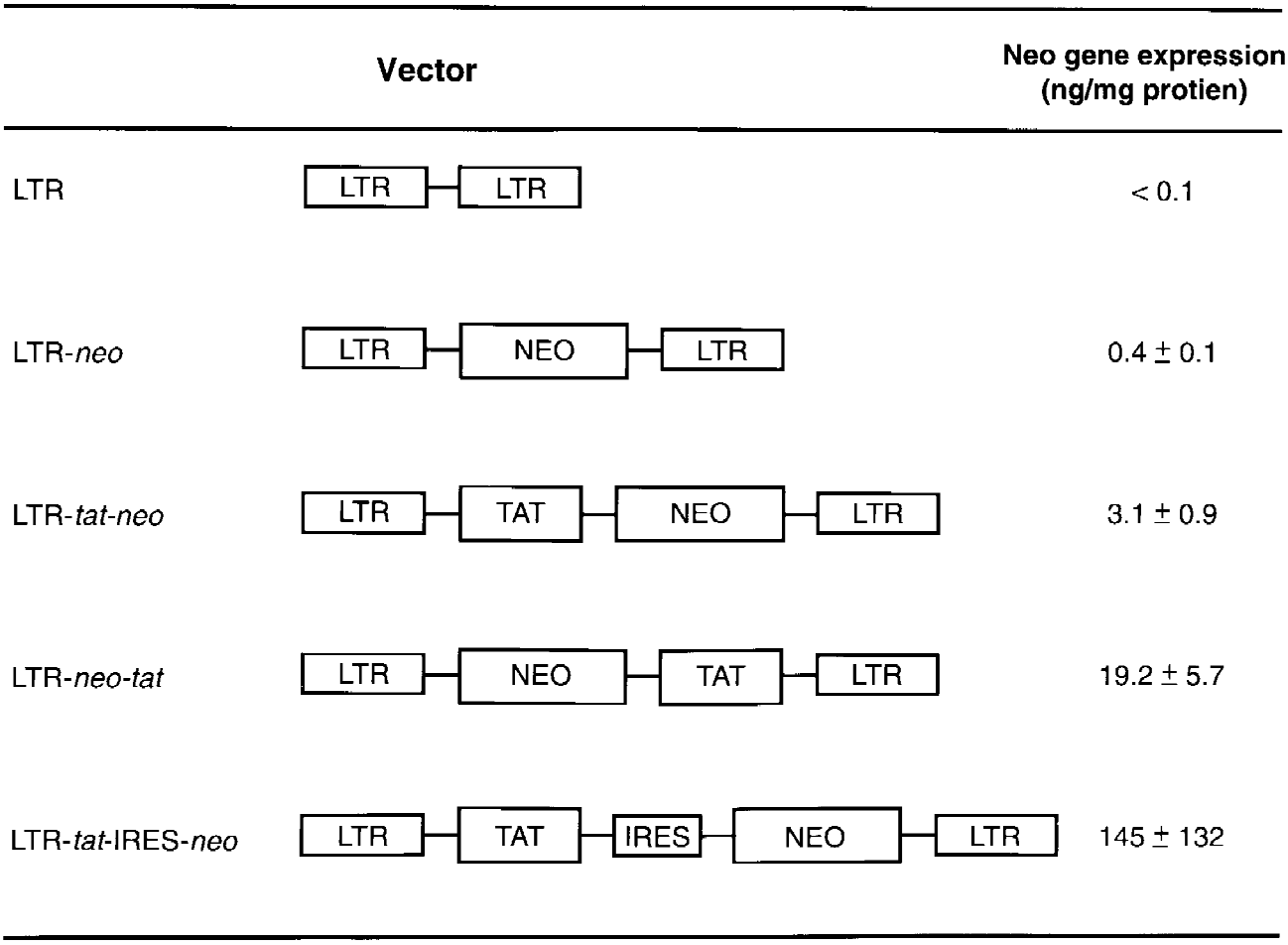


Fig. 5. Expression of *neo* gene in HIV-2 LTR-based dual gene vectors in human lymphocytic CEM cells in long-term culture. About 6×10^6 cells were transfected with 6 μg of retroviral vector DNA by electroporation. About 40 to 48 hr postinfection, the transfected cells were cultured in the presence of G418 (400 μg/ml) and cultures were maintained thereafter in G418-containing medium. For biochemical analysis, aliquots of cultures were harvested at day 28 after drug selection, and aliquots of the cellular extracts were assayed for Neo protein by the commercially available ELISA as described in Materials and Methods.

Notably, the magnitude of transactivation and of positional effects were also cell-type dependent, a phenomenon apparently not fully appreciated in previous studies. The *tat* gene in LTR-*tat* was less effective in transactivating HIV-2 LTR-directed CAT gene expression in epithelioid COS than in lymphoid CEM cells. It is possible that a lesser amount of Tat is produced in COS than in CEM cells. The expression of this gene is being directed by the LTR which is subject to modulation by several cellular transcriptional factors. It contains a multitude of DNA binding domains upstream of the transcriptional initiation site, some common with other cellular promoters and others more distinct [Markovitz et al., 1990; Arya, 1990]. Availability or abundance of such cellular factors would affect basal level expression of the LTR directing *tat* gene and this may be cell type dependent. Alternatively, Tat, itself requiring cellular factors, once produced may be less effective in transactivating the LTR in COS cells than in CEM cells. Tat employs two different mechanisms for transactivation-enhancement of transcriptional initiation and promotion of transcript elongation [Laspia et al., 1989]. It is possible that while in lymphocytic cells both mechanisms are effective, only one of them is operative in epithelioid cells. Similarly, the effect of the position of the gene on its expression was more modest in COS cells than in CEM cells. Given that the transcriptional and translational apparatuses engage a myriad of cellular factors, which may be more limiting in one cell type than the other, the cell-type dependent differential expression is perhaps not surprising.

High level expression of the downstream gene can be achieved either by providing a mechanism for independent translational initiation (translational enhancement) or by including an internal promoter to obtain independent transcription (transcriptional enhancement). The placement of IRES sequence between the *tat* and *neo* genes indeed enhanced the expression of the downstream *neo* gene. Thus, even in *tat*-transactivated LTR-based vectors, provision of internal translational initiation site appeared to be beneficial. In contrast, the usefulness of using internal promoter

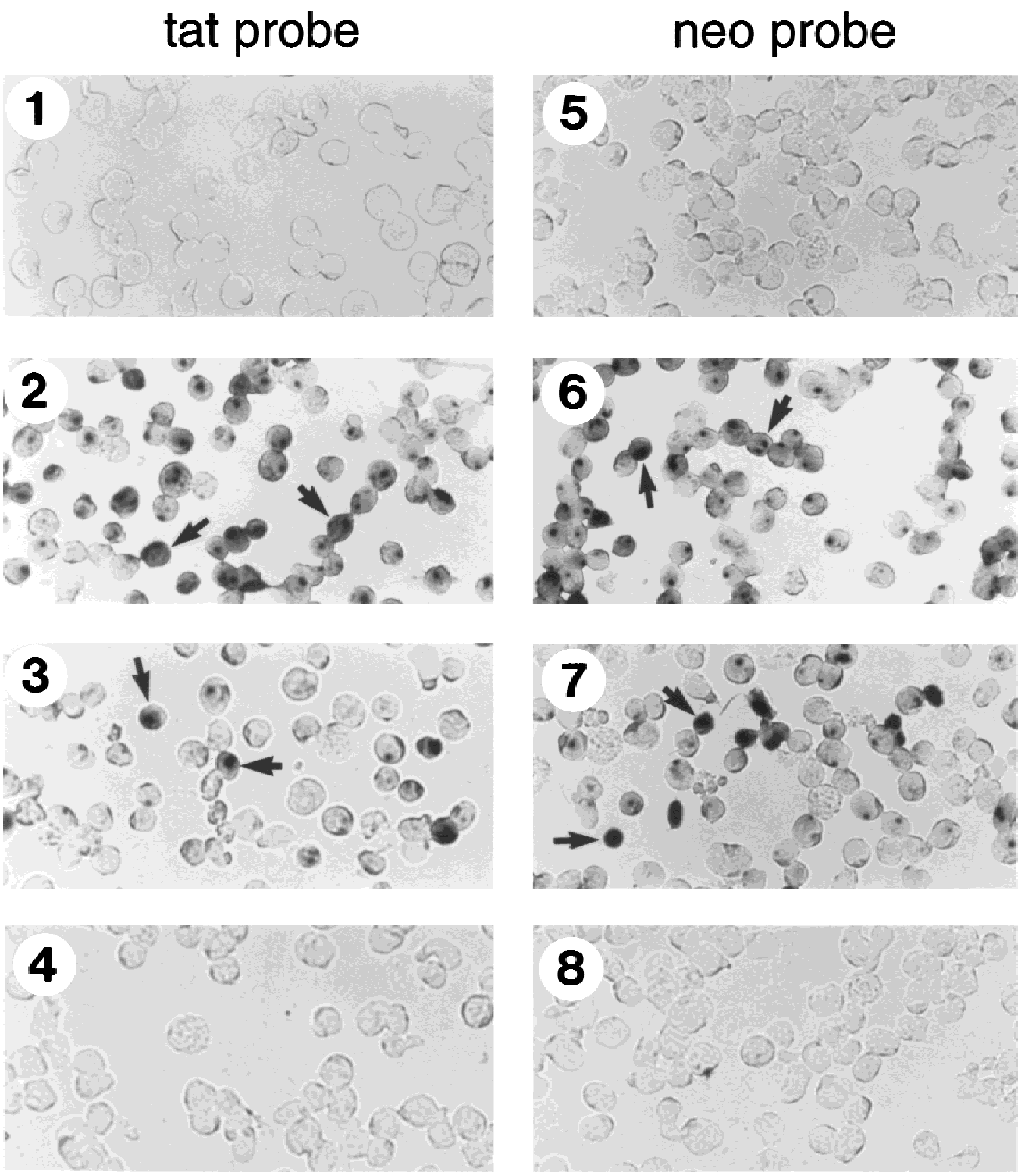


Fig. 6. Visualization of CEM cells expressing *tat* and *neo* genes by in situ hybridization. Cells were transfected and maintained under drug selection as described in the legend to Figure 5. At 28 days postselection, cultures were stained with antisense *tat* (**1–4**) and *neo* (**5–8**) gene probes labeled with digoxigenin in vitro. Arrows indicate examples of positive cells. Panels 1 and 5, LTR-*neo;* panels 2 and 6, LTR-*tat*-*neo;* and panels 3 and 7, LTR-*neo*-*tat*. Panels 4 and 8 are for control cultures incubated with antisense *tat* and *neo* probes, respectively.

in the presence of LTR: Tat transactivation loop was not evident in this study. The prototype heterologous viral (CMV) and cellular (β-actin) promoters tested here were both orders of magnitude less effective in directing gene expression than the LTR-Tat combination. We suspect that in a vector that is designed to direct the expression of the downstream gene with an internal promoter, but in the context of LTR:Tat, the

TABLE I. Comparative Activity of Promoters in Human T Lymphocytic CEM Cells

	Relative CAT gene expression	
Promoter-indicator	No HIV-2	+HIV-2
HIV-2 LTR-CAT	1.0	1,577 ± 834
CMV-promoter-CAT	280 ± 36	84 ± 16
β-Actin promoter-CAT	8.2	1.2 ± 0.4

expression of both genes directed by the LTR will continue to occur and may even predominate. The possible complication due to promoter interference would be an additional factor to consider. An interesting idea was to use a constitutive promoter, but make it responsive to transactivation by Tat by inserting a Tat-response (TAR) element into the promoter. However, the results of the recent studies with the chimeric promoters between HIV-1 TAR element and Rous sarcoma virus (RSV) LTR [Mukhtar et al., 1996] and cytomegalovirus (CMV) promoter [Robinson et al., 1995] have not been encouraging.

In summary, our results establish the feasibility of designing HIV-2-based multigenic vectors. The exploitation of the LTR:Tat regulatory loop used in this study is only one example of the regulatory network possessed by HIV-2 and other lentiviruses. Combined with a suitable packaging system, these vectors will enable high level but regulated expression of the transgene. We are developing homologous (Tat-regulated) and heterologous (tetracycline-induced) human packing cell lines for this purpose. In addition, these vectors can also be packaged into liposomes [Arya and Gallo, 1996; Al-Harthi, Owais and Arya, submitted] to target a wide variety of cells. If HIV-2 can itself inhibit HIV-1 in vivo, as it does in vitro, that will be an added bonus complementing the effect of the transgene. Several transgenes are being evaluated for gene therapy of AIDS. These include transdominant Rev-M10 [Woffendin et al., 1996], anti-*rev* antibody gene [Duan et al., 1997], targeted ribozymes [Gervaix et al., 1997], and anti-Tat gene [Rosenzweig et al., 1997]. Any one of those and other protective genes would be good candidates for including in HIV-2-based vectors. In addition, HIV-2 vector DNA can be directly used for in vivo DNA vaccination as has been demonstrated for other vector DNAs [Boyer et al., 1997]. The inherent advantages of HIV-2-derived vectors ought to be considered while other efforts are directed to alternate approaches. For example, adenoviruses which act extra-chromosomally result only in temporary expression of therapeutic genes and are usually eliminated because of the host immune response. Thus, lentivirus vectors would appear to be the vector of choice for long-term expression and targeting of nondividing hematopoietic stem cells and neuronal cells.

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REFERENCES

Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen, ISY (1996): High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein. Journal of General Virology 70:2581–2585.

Anderson WF (1992): Human gene therapy. Science 256:808–813.

Arya SK (1987): 3′-*orf* and *sor* genes of human immunodeficiency virus: In vitro transcription-translation and immunoreactive domains. Proceedings of the National Academy of Sciences USA 84: 5429–5433.

Arya SK, Gallo RC (1988): Human immunodeficiency virus type 2 long terminal repeat: Analysis of regulatory elements. Proceedings of the National Academy of Sciences USA 85:9753–9757.

Arya SK (1990): Human immunodeficiency virus type-2 gene expression: Two enhancers and their activation by T-cell activators. New Biologist 2:57–65.

Arya SK (1993): Human immunodeficiency virus type 2 (HIV-2) transactivator (Tat): Functional domains and the search for transdominant negative mutants. AIDS Research and Human Retroviruses 9:839–848.

Arya SK, Sadaie MR (1993): Fusogenicity of mutant and chimeric proviruses derived from molecular clones of cytopathic and noncytopathic human immunodeficiency virus type 2. Journal of Acquired Immune Deficiency Syndromes 6:1205–1211.

Arya SK, Mohr JR (1994): Conditional regulatory elements of human immunodeficiency virus type 2 long terminal repeat. Journal of General Virology 75:2253–2260.

Arya SK, Zamani M, Gallo RC (1994): HIV-2 inhibits HIV-1. AIDS Research and Human Retroviruses 10:S158.

Arya SK, Gallo RC (1996a): Advances in genetics: Gene therapy. In Lanza R (ed): "One World: The Health and Survival of the Human Species in the 21st Century." New Mexico: Health Press, pp 49–57.

Arya SK, Gallo RC (1996b): Human immunodeficiency virus (HIV) type-2 mediated inhibition of HIV type 1: A new approach to gene therapy of HIV infection. Proceedings of the National Academy of Sciences USA 93:4486–4491.

Boyer JD, Ugen KE, Wang B, Agadjanyan M, Gilbert L, Bagarazzi ML, Chattergoon M, Frost P, Javadian A, Williams WV, Rafadi Y, Ciccarelli RB, McCallus D, Coney L, Weiner DB (1997): Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. Nature Medicine 3:526–532.

Bowtell DD, Cory S, Johnson GR, Gonda TJ (1988): Comparison of expression in hemopoietic cells by retroviral vectors carrying two genes. Journal of Virology 62:2464–2473.

Chen BF, Hwang LH, Chen DS (1993): Characterization of a bicistronic retroviral vector composed of the swine vesicular disease virus internal ribosome entry site. Journal of Virology 67:2142–2148.

Corbeau P, Kraus G, Wong-Staal F (1996): Efficient gene transfer by a human immunodeficiency virus type I (HIV-I)-derived vector utilizing a stable HIV packaging cell line. Proceedings of the National Academy of Sciences USA 93:14070–14075.

Correll PH, Colilla S, Karlsson S (1994): Retroviral vector design for long-term expression in murine hematopoietic cells in vivo. Blood 84:1812–1822.

Crystal RB (1995): Transfer of genes to humans: Early lessons and obstacles to success. Science 270:404–410.

Duan L, Zhang H, Oakes JW, Bagasra O, Pomerantz RJ (1997): DNA and amino acid sequence of anti-HIV-1 Rev sFV. Human Gene Therapy 8:510–516.

Fletcher TM, Brichacek B, Sharova N, Newman MA, Stivahtis G,

Sharp PM, Emerman M, Hahn BH, Stevenson M (1996): Nuclear import and cell cycle arrest functions of the HIV-1 vpr protein are encoded by two separate genes in HIV-2/SIVsm. EMBO Journal 15:6155–6165.

Garzino-Demo A, Gallo RC, Arya SK (1995): Human immunodeficiency virus type 2 (HIV-2): Packaging signal and associated negative regulatory element. Human Gene Therapy 6:177–184.

Gervaix A, Li X, Kraus G, Wong-Staal F (1997): Multigene antiviral vectors inhibit diverse human immunodeficiency virus type 1 clades. Journal of Virology 71:3048–3053.

Ghattas IR, Sanes JR, Majors JE (1991): The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. Molecular and Cellular Biology 11:5848–5859.

Gilboa E, Eglitis MA, Kantoff PW, Anderson WF (1986): Transfer and expression of cloned genes using retroviral vectors. Biotechniques 4:504–512.

Kashanchi F, Melpolder JC, Epstein JS, Sadaie MR (1997): Rapid and sensitive detection of cell-associated HIV-1 in latently infected cell lines and in patient cells using sodium-n-butyrate induction and RT-PCR. Journal of Medical Virology 52:179–189.

Kozak M (1991): Structural features in eukaryotic mRNAs that modulate the initiation of translation. Journal of Biological Chemistry 266:19867–19870.

Kumar P, Hui H, Kappes TC, Haggarty BS, Hoxie JA, Arya SK, Shaw GM, Hahn BH (1990): Molecular characterization of an attenuated human immunodeficiency virus type 2 isolate. Journal of Virology 64:890–901.

Laspia MF, Rice AP, Matthews MP (1989): HIV-1 tat protein increases transcriptional initiation and stabilizes elongation. Cell 59:283–292.

Li M, Hantzopoulos PA, Banerjee D, Zhao SC, Schweitzer BI, Gilboa E, Bertino JR (1992): Comparison of the expression of a mutant dihydrofolate reductase under control of different internal promoters in retroviral vectors. Human Gene Therapy 3:381–390.

Markovitz DM, Hannibal M, Perez VL, Guantt C, Folks TM, Nabel GJ (1990): Differential regulation of human immunodeficiency viruses (HIVs): A specific regulatory element in HIV-2 responds to stimulation of the T-cell antigen receptor. Proceedings of the National Academy of Sciences USA 87:9098–9102.

Miller AD, Rosman GJ (1989): Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980–982, 984–986, 989–990.

Mitchell BS, Dhani D, Schumacher U (1992): In situ hybridization: A review of methodologies and applications in biomedical sciences. Medical Laboratory Sciences. 49:107–118.

Morgan RA, Couture L, Elroy-Stein O, Ragheb J, Moss B, Anderson WF (1992): Retroviral vectors containing putative internal ribosome entry sites: Development of a polycistronic gene transfer system and applications to human gene therapy. Nucleic Acids Research 20:1293–1299.

Mukhtar M, Duan L, Bagasra O, Pomerantz RJ (1996): Evaluation of relative promoter strengths of the HIV-1-LTR and a chimeric RSV-LTR in T lymphocytic cells and peripheral blood mononuclear cells: Promoters for anti-HIV-1 gene therapies. Gene Therapy 3: 725–730.

Mulligan RC (1993): The basic science of gene therapy. Science 260: 926–932.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996): In vivo gene delivery and stable transduction of non-dividing cells by a lentiviral vector. Science 272:263–267.

Robinson D, Elliot JF, Chang L-J (1995): Retroviral vector with a CMV-IE/HIV-1 TAR hybrid LTR gives high basal expression levels and is up-regulated by HIV-1 Tat. Gene Therapy 2:269–278.

Rosenzweig M, Marks DF, Hempel D, Lisziewicz J, Johnson RP (1997): Transduction of CD34+ hematopoietic progenitor cells with an antistat gene protects T-cell and macrophage progeny from AIDS virus infection. Journal of Virology 71:2740–2746.

Woffendin C, Ranga U, Yang Z, Xu L, Nabel GJ (1996): Expression of a protective gene prolongs survival of T-cells in human immunodeficiency virus-infected patients. Proceedings of the National Academy of Sciences USA 93:2889–2894.